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Karolinska Institutet, Stockholm, Sweden

BETA-ARRESTINS IN CANCER: LINKING PRO-TUMORIGENIC EXTRACELLULAR ACTIVATED SIGNALING WITH THE TUMOR SUPPRESSOR P53 PATHWAY

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Stockholm 2017

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Published by Karolinska Institutet.
Printed by E-Print AB, 2017

ISBN 978-91-7676-731-3

BETA-ARRESTINS IN CANCER: LINKING PRO-TUMORIGENIC EXTRACELLULAR ACTIVATED SIGNALING WITH THE TUMOR SUPPRESSOR P53 PATHWAY

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

The IGF-1R is an important player in cancer development that maintains the malignant phenotype by inducing cell proliferation, survival, transformation, motility and invasiveness. Activation of IGF-R results in its Mdm2-dependent ubiquitination and degradation followed by MAPK signaling. IGF-1R ubiquitination by Mdm2 is mediated by scaffolding protein β-arrestin 1. The tumor suppressor p53 pathway is activated in damaged cells causing growth arrest and if necessary, apoptosis and senescence. In normal conditions p53 is inactivated by Mdm2. Activation of extracellular pro-survival signaling has been shown to inhibit p53 activity through β-arrestin 1. Thus the same Mdm2/β-arrestin 1 system regulates two important pathways involved in cancer.

The aim of this thesis was to investigate in detail the IGF-1R/β-arrestin/Mdm2/p53 axis and explore the potential use of its components as anti-cancer therapeutic targets.

In **Paper I** we analysed the molecular interplay between p53 and IGF-1R through Mdm2. We tested the effect of p53/Mdm2 disruption on IGF-1R using a panel of melanoma cells and the p53-rectivator Nutlin-3. Disruption of the p53/Mdm2 interaction by Nutlin-3 increased the IGF-1R/Mdm2 interaction, followed by IGF-1R degradation and MAPK activation. This resulted in reduced cell proliferation and invasion and had a two-step effect on cell migration, demonstrating that modulation of the p53/Mdm2/IGF-1R axis is a potential anti-cancer therapeutic strategy.

In **paper II** we focused on the role of β-arrestin isoforms in the p53/Mdm2/IGF-1R axis. By modulating levels of β-arrestin 1 or 2 we identified opposing roles of isoforms on IGF-1R degradation, signaling and p53 pathway. We revealed a higher affinity of ligand-free IGF-1R for β-arrestin 2, and ligand occupied receptor - for β-arrestin 1. Antagonism between isoforms was also observed on biological effects with β-arrestin 2 causing cell cycle arrest and inhibiting IGF-1 response and cell viability, and β-arrestin 1 acting in the opposite direction. Thus we identified the β-arrestin 1/2 system as a second potential drug target within the p53/Mdm2/IGF-1R axis.

In **paper III** we studied the possibility of co-targeting the p53/Mdm2/IGF-1R and the MAPK pathway in melanoma cell lines. We combined MEK inhibitors with 1) balanced IGF-1R inhibition by siRNA; 2) biased IGF-1R inhibition by Nutlin-3, inducing transient MAPK; and 3) biased IGF-1R inhibition by antibody CP, inducing prolonged MAPK. We identified strong synergy between Nutlin-3 and MEK inhibitors. This combination of specific biased IGF-1R inhibition with MEK inhibitors is the first rational anti-cancer strategy identified in this thesis.

In **paper IV** we investigated the possibility of co-targeting the β-arrestin system with the DNA-damage inducing drug dacarbazine. By modulating the level of β-arrestin isoforms in melanoma cell lines we demonstrated that both β-arrestin 1 inhibition and β-arrestin 2 overexpression synergize with dacarbazine. This study revealed the second rational anti-cancer strategy of this project.

To sum up, our findings demonstrate that the p53/Mdm2/IGF-1R axis is a potential target for anti-cancer therapy. However, optimal effects can be achieved only through accurate modulation of multiple pathways regulated by the axis.

LIST OF SCIENTIFIC PAPERS

I. Worrall C, **Suleymanova N**, Crudden C, Trocoli Drakensjö I, Candrea E, Nedelcu D, Takahashi S-I, Girnita L and Girnita A. Unbalancing p53/Mdm2/IGF-1R axis by Mdm2 activation restrains the IGF-1-dependent invasive phenotype of skin melanoma
Oncogene 2017 Jun 8;36(23):3274-3286; doi: 10.1038/onc.2016.472
PMID: 28092675

II. **Suleymanova N**, Crudden C, Shibano T, Worrall C, Oprea I, Tica A, Calin GA, Girnita A and Girnita L. Functional antagonism of β-arrestin isoforms balance IGF-1R expression and signaling with distinct cancer-related biological outcomes
Oncogene doi: 10.1038/onc.2017.179; Epub ahead of print 5 June 2017
PMID: 28581517

III. **Suleymanova N***, Crudden C*, Worrall C, Dricu A, Girnita A and Girnita L. Enhanced response of melanoma cells to MEK inhibitors following unbiased IGF-1R down-regulation
Oncotarget 2017 In press

IV. **Suleymanova N**, Crudden C, Gebhard B, Girnita L, Girnita A. Competing engagement of β-arrestin isoforms balance IGF-1R signaling and control response of melanoma cells to chemotherapy
Manuscript

* Equal contribution

RELATED PUBLICATIONS

I. Crudden C, Ilic M, **Suleymanova N**, Worrall C, Girnita A, Girnita L. The dichotomy of the Insulin-like growth factor 1 receptor: RTK and GPCR: friend or foe for cancer treatment?
Growth Horm IGF Res. 2015 Feb;25(1):2-12.
doi: 10.1016/j.ghir.2014.10.002. Epub 2014 Oct 28. Review.
PMID: 25466906

II. Worrall C, Nedelcu D, Serly J, **Suleymanova N**, Oprea I, Girnita A, Girnita L. Novel mechanisms of regulation of IGF-1R action: functional and therapeutic implications.
Pediatr Endocrinol Rev. 2013 Jul;10(4):473-84. Review.
PMID: 23957198

CONTENTS

LIST OF ABBREVIATIONS

A-loop	Activation loop
ALL	Acute lymphoblastic leukemia
AP2	Adaptor protein 2
AR	Androgen receptor
ARF	ADP-ribosylation factor
AT1R	Angiotensin II type 1 receptor
ATP	Adenosine triphosphate
BAD	Bcl-2-associated death promoter
Bcl-2	B-cell lymphoma 2
Bcl-X1	B-cell lymphoma-extra large
BRCA1	Breast cancer gene 1
Cas	Crk-associated substrate
CML	Chronic myelogenous leukemia
CXCL12	C-X-C Motif Chemokine Ligand 12
CXCR	C-X-C chemokine receptor
Cytochrome C	Cytochrome complex
D1A	Dopamin 1A
DNA	Deoxyribonucleic acid
DOR	δ opioid receptor
DTIC	Dacarbazine
ECM	Extracellular matrix
EDG1	Endothelial differentiation gene 1
EDG2	Endothelial cell differentiation gene-2
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinases
ETAR	Endothelin A receptor
FACS	Fluorescence-activated cell sorting
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FPR1	Formyl peptide receptor 1
GBM	Glioblastoma
GDP	Guanosine-5'-diphosphate
GEF	Guanine exchange factor
GPCR	G-protein-coupled receptor
Grb2	Growth factor receptor-binding protein 2
GRK	G protein-coupled receptor kinase
GSK-3β	Glycogen synthase kinase 3β
GTP	Guanosine-5'-triphosphate
HEK	Human embryonic kidney cells
HIF-1α	Hypoxia-inducible factor 1α
IGF-1/2	Insulin-like growth factor 1/2
IGF-1R/IGF-2R	Insulin-like growth factor 1/2 receptor
IGFBP	Insulin-like growth factor-binding protein
IP	Immunoprecipitation
IR	Insulin receptor
IRS	Insulin receptor substrate

JNK3	c-Jun N-terminal kinase 3
KISS1R	KiSS1-derived peptide receptor
KO	Knockout
LPAR	Lysophosphatidic acid receptor
MAPK	Mitogen-activated protein kinase
MBM	Medulloblastoma
Mdm2	Mouse double minute 2 homolog
MEF	Mouse embryonic fibroblast
MEK	MAPK/ERK kinase
MMP	Matrix metallopeptidase
MOR	μ opioid receptor
N1	Neurotensin 1
nAChR	Nicotinic acetylcholine receptor
Nedd4	Neuronal precursor cell-expressed developmentally downregulated 4
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK1R	Neurokinin-1 receptor
p125Fak	p125 focal adhesion kinase
p130Cas	p130 Crk-associated substrate
pAkt	Phospho-Akt
PAR1/2	Protease-activated receptors 1/2
PDE	Phosphodiesterase
PDGFβ	Platelet-derived growth factor β
PDGFR	Platelet-derived growth factor receptors
PDK1	3-phosphoinositide-dependent protein kinase 1
pERK	Phospho-ERK
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PIP2	Phopsphatidyl-inositol bisphosphate
PIP3	Phopsphatidyl-inositol trisphosphate
PKCε	Protein kinase C ε
PLCγ	Phosphoinositide phospholipase C γ
PPP	Picropodophyllin
pRb	Retinoblastoma protein
PTB	Phosphotyrosine-binding domains
pVHL	von Hippel-Lindau tumor suppressor
RTK	Receptor tyrosine kinase
S412	Serine-412
SH2	Src homology region 2 domain
Shc	Src homology 2-containing protein
Sos	Son of sevenless
SREBP	Sterol regulatory element-binding protein
TK	Tyrosine Kinase
TP53	Tumor protein 53
TPβR	Thromboxane A2 β-type receptor
TrkA	Tropomyosin receptor kinase A
TrkB	Tropomyosin receptor kinase B
TSP1	Thrombospondin 1
TβRIII	TGF-β co-receptor type III
uPA	Urokinase plasminogen activator

uPAR	Urokinase plasminogen activator receptor
V2	Vasopressin 2
V2R	Vassopressin 2 receptor
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
WB	Western blot
WT1	Wilms' tumour suppressor 1
α1AR	α1 adrenergic receptor
α2AR	α2 adrenergic receptor
β-arr	β-arrestin
β1AR	β1 adrenergic receptor
β2AR	β2 adrenergic receptor

1. INTRODUCTION

1.1. Cancer and its hallmarks

Cancer is one of the leading causes of morbidity worldwide, accounting for approximately 13% of all deaths each year [1]. The number of diagnosed cases of cancer increases every year, particularly in countries with an ageing population [1]. More than 30% of cancer deaths could be avoided by better control of risk factors, early diagnosis and treatment [2-5].

Cancer development is a complex and multi-step process during which initially normal cells acquire various capabilities to grow, survive and disseminate [6]. These capabilities include six core and two emerging hallmarks of cancer [6].

Core hallmarks of cancer include:

(i) **Sustaining proliferative signaling:** In normal cells entry into a new cycle is under the control of growth factors, such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1) etc [7]. In cancer cells these pathways are dysregulated by the increased production of growth factors or receptors as well as by structural changes of the receptor and/or components of its downstream signaling [8-10].

(ii) **Evading growth suppressors:** Cancer cells also have to bypass multiple tumor suppressor pathways that limit proliferation of normal cells [11]. Tumor suppressor proteins such as retinoblastoma protein (pRb) and p53 are able to induce cell cycle arrest or apoptosis in response to various extra- and intracellular signals [12, 13]. In cancer cells these proteins can be lost or mutated and gain new carcinogenic functions [14, 15].

(iii) **Resisting cell death:** Normal cells in stressed conditions undergo cell death via apoptosis, autophagy and necrosis through the p53 pathway [16, 17]. Tumor cells can avoid apoptosis through increased proliferative signaling or modulation of anti-apoptotic and pro-apoptotic factors [18]. However, tumor cells can also induce cell death in order to recruit carcinogenic inflammatory agents [19, 20].

(iv) **Enabling replicative immortality:** Chromosomal DNA of cells is protected by telomeres that shorten in time increasing possibilities of chromosomal fusions, leading to cell senescence and apoptosis [21]. Cancer cells are able to conserve telomere length, by increased production of the protective enzyme telomerase [22-25].

(v) **Inducing angiogenesis:** In normal adult tissues, angiogenesis is activated transiently in accordance with physiological needs [26]. In cancer cells it is constantly activated by dysregulation of pro-angiogenic growth factors, such as VEGF, fibroblast growth factor (FGF) and thrombospondin 1 (TSP1), sustaining a tumor's nutrient supply and providing a transport system for metastasizing cells [27-29]. Moreover, the angiogenic switch is also facilitated by infiltration of tumor by cells of the immune system [30, 31].

(vi) **Activating invasion and metastasis:** Normally cells are attached to each other as well as to the extracellular matrix [32]. Cancer cells can lose these abilities by

expression of embryonic factors enabling epithelial-mesenchymal transition and dysregulation of adhesion factors enabling motility and invasiveness [33, 34]. Immune cells surrounding the tumor can contribute by activating proteolytic enzymes or growth factors [35, 36]

Emerging hallmarks of cancer include:

(vii) **Reprogramming energy metabolism:** Normal cells undergo two stages of glycose processing and switch to the single stage glycolysis in anaerobic conditions [37]. Cancer cells can switch to the single stage glycolysis even in the presence of the oxygen thus possibly redirecting glycolytic intermediates towards neighboring and new cells [38-40]. This switch can be induced by activation of oncogenes or alteration of tumor suppressors [41, 42].

(viii) **Evading immune destruction:** Cells are constantly scanned by immune cells and eliminated in case of aberrancy [43, 44]. Cancer cells acquire the ability to hide and evade immune system by producing immunosupressive factors or recruiting immunosupressive inflammatory cells [45-48].

Two enabling characteristics of cancer are required for the acquisition of hallmark of cancer by normal cells [6, 49]. These characteristics include:

(i) **Genome instability and mutation:** Various alterations in the cellular genome enable acquisition of the hallmarks of cancer [50]. Mutations of certain genes involved in guarding DNA, such as p53, facilitate further aquisition of carcinogenic mutations and survival of transformed cells [25, 51].

(ii) **Tumor promoting inflammation:** Inflammation and recruitment of the immune cells also helps to acquire hallmarks of cancer by supplying cells with various survival, growth and proangiogenic factors as wells as mutagenic oxygen species [20, 52, 53].

In addition, the **tumor microenvironment** can exploit neighboring normal cells, creating beneficial surroundings for tumor growth thus acting as a contributing factor to cancer [35, 54, 55].

Various reconsideration of classification were proposed such as including such characteristics as genome instability and stress phenotypes as a separate emerging hallmarks of cancer [56].

1.2. Growth factors and tumor suppressors

The balance between two important biological pathways are involved in most if not all hallmarks of cancer: growth factor receptor signaling and tumor suppressor pathways [57-61].

Growth factors bind to their receptors, activating signaling pathways that regulate proliferation, survival and differentiation of normal cells [62-65]. These pathways become potentially oncogenic when any of their components are hyper-activated due to various genetic or epigenetic alterations [8, 66, 67].

The anti-oncogenic **tumor suppressors** negatively regulate cell proliferation and normally induce death of incompatible cells [61]. This pathway becomes oncogenic when its components

lose their activity as a result of various mutations [61, 68]. Typically both copies of tumor suppressor genes must be mutated for malignant transformation of cell [69].

1.3. Growth factor receptors and cancer

Most of the growth factor receptors belong to the family of receptor tyrosine kinases (RTKs) [10, 70]. The RTK family includes 58 receptors with related structure [71, 72]. Most RTKs are monomers with extracellular, transmembranous and intracellular domains with protein kinase activity [10, 73]. Ligand binding to the extracellular domain induces RTK dimerization [10, 71, 73]. Dimerized monomers cross-phosphorylate tyrosine resides in the intracellular domains of each other, increasing the receptor-kinase activity and creating docking sites for the proteins involved in signal transduction [73, 74]. Various downstream molecules containing SH2 or PTB domains are directly or indirectly recruited to these phosphotyrosines, transmitting information to the cell's nucleus and other regulatory organelles [71, 75, 76]. The most well studied examples of activation cascades downstream of RTKs are the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinases (PI3Ks) pathways [77].

Increased production or hyperactivation of different component of RTK signaling contributes to cancer development [9, 10, 36, 78]. Some of the RTKs involved in cancer development include epidermal growth factor receptors (EGFR), vascular endothelial growth factor receptors (VEGFR), platelet-derived growth factor receptors (PDGFR), fibroblast growth factor receptor (FGFR) and insulin-like growth factor receptor IGF-1R [9, 10].

The EGFRs regulate proliferation, differentiation and migration of normal cells [79]. Increased production of ligands and/or receptors as well as mutations of the tyrosine kinase domain are found in diverse types of cancers and associated with poor prognosis [10, 80].

VEGFR is mainly expressed on endothelial cells and plays a key role in vasculo-, angio- and lymphoangiogenesis [81, 82]. VEGFRs contribute to carcinogenesis by formation of blood and lymph vessels in the tumor and its microenvironment [83]. Up-regulation of VEGFR is found in various types of malignancies [84].

PDGFR normally controls proliferation and migration of mesenchymal cells [85]. Autocrine signaling maintained by this receptor has been demonstrated in many tumors [86, 87].

FGFRs are involved in organ development during embryogenesis as well as in tissue homeostasis, proliferation, inflammation and angiogenesis in adults [28, 65]. Gene amplification, chromosomal translocations, gene fusions and missense mutations of FGFRs are found in various types of cancers [28].

IGF-1R system

The IGF-1R system consists of two ligands (IGF-1 and IGF-2), three cell membrane receptors (IGF-1R, IGF-2R and IR) and seven binding proteins (IGFBP-1-7) [88]. IGF-1 and IGF-2 ligands share 62% homology and are mainly secreted by hepatocytes after growth hormone stimulation [89]. IGF-1 ligand in turn inhibits the secretion of growth hormone by the pituitary glands [90]. Around 90% of circulating IGF is inactive through binding to IGFBPs [91, 92].

IGF-1R structure

IGF-1R belongs to the family of insulin receptors with a structure more complex than most RTKs [73, 74, 93]. The IGF-1R gene encodes for a single precursor that is cleaved generating two α and two β subunits [94]. Distinct subunits are heterodimerized after glycosylation further forming a tetramer stabilized by disulfide bonds (β-α-α-β) [94, 95]. α-subunits are entirely extracellular and contain an IGF-1 binding site, while β subunits span the membrane and consist of extracellular, transmembranous and intracellular parts [93, 95]. The intracellular part of the β-subunit is divided into a juxtamembrane domain, tyrosine kinase domain and C-terminal domain [96].

IGF-1R function

In an inactive state the activation loop (A-loop) of the intracellular tyrosine kinase domain of IGF-1R behaves as a pseudo-substrate, blocking both substrate and ATP-binding sites of the receptor [97]. IGF-1 and IGF-2 binding to the α-subunit of IGF-1R causes conformational changes in the receptor followed by autophosphorylation of tyrosines at positions 1131, 1135 and 1136 of the A-loop by their dimeric subunit partner [71, 97]. Initial phosphorylation of Y1135 induces a stable structure followed by phosphorylation Y1131 and Y1136 [97, 98]. These conformational changes of the A-loop allow ATP binding and activation of several other tyrosine residues including Y950 at juxtamembrane domain of the receptor [97, 98]. Activated Y950 serves as a docking site for insulin receptor substrate 1 (IRS1) and Src homology 2-containing protein (Shc) that link the activated receptor to downstream pathways activating tyrosine kinase signaling (TK signaling) of IGF-1R [97, 98]. (Figure 1)

IRS-1 binds to the regulatory p85 subunit of PI3Ks, which allows the activation of its catalytic p110 subunits [99]. Activated PI3K phosphorylates the phosphatidyl-inositol bisphosphate (PIP2) and generates phosphatidyl-inositol trisphosphate (PIP3), which interacts with serine threonine kinase Akt [95, 99]. This interaction causes translocation of the complex to the inner membrane and activation of Akt by 3-phosphoinositide-dependent protein kinase 1 (PDK1) [100, 101]. Activated Akt induces numerous cellular processes including cell cycle progression, cell migration and anti-apoptosis signals [99, 102]. (Figure 1)

Shc or IRS-1 also recruits the growth factor receptor-binding protein 2 (Grb2) that interacts with son of sevenless (SOS), a plasma membrane guanine exchange factor (GEF) for Ras protein [103, 104]. Activated Ras interacts with Raf, which phosphorylates and activates MAPK/ERK kinase (MEK), which in turn phosphorylates and activates extracellular signal-regulated kinases (ERKs) [88, 105]. This signaling cascade is called MAPK pathway [106]. Activated ERKs phosphorylate a number of cytosolic substrates and translocate to the nucleus, activating various transcription factors that regulate cellular proliferation, differentiation and transformation [107, 108]. (Figure 1)

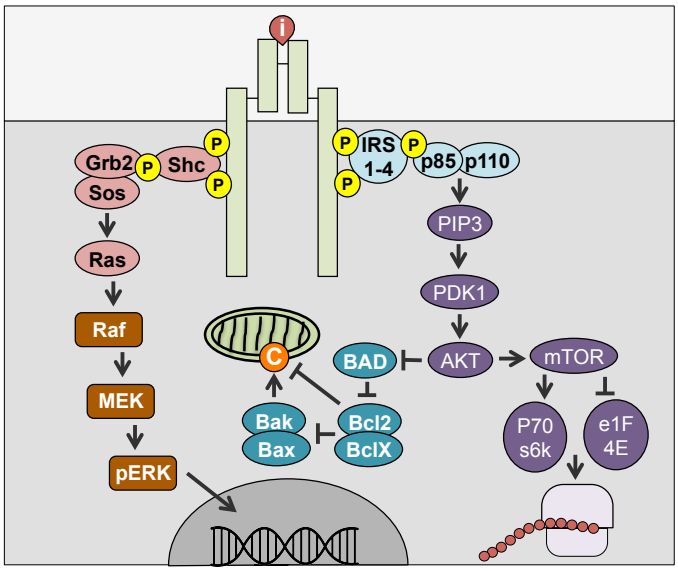

Figure 1. *Schematic illustration of signaling cascades downstream of IGF-1R: Activated IGF-1R recruits 1) Shc inducing MAPK pathway, which leads to the transcriptional activation of genes involved in cell proliferation, transformation and differentiation; and 2) IRS1-4 inducing PI3K pathway, involved in protein synthesis through mTOR and anti-apoptosis through inhibition of BAD.*

Biological effects

The IGF-1R axis has been shown to play important roles in neonatal and pubertal development by inducing cell proliferation, differentiation and inhibiting apoptosis [109]. It is also important for maintenance of normal cell growth, metabolism and adhesion [110, 111]. Disruption of IGF-1 and IGF-2 genes result in dwarfism, decreased viability and significant weight loss in mice, that survive, while disruption of the IGF-1R gene results in a more severe dwarfism and death shortly after birth [112]. Double disruption of both IGF-1 and IGF-1R genes results in a phenotype similar to that seen in the disruption of IGF-1R, while double disruption of IGF-2 with IGF-1R and IGF-1 with IGF-2R genes results in a further increase in dwarfism [112].

The first report of IGF-1R involvement in cancer was by Pollak *et al.* in 1987 [113]. Since then a growing body of evidence indicates critical roles for the IGF-1R axis in cancer [111]. Various components of IGF-1R axis are elevated during cancer development [111, 114, 115]. Increased level of IGF-1 and IGF-2 ligands and IGF-1R were found in various types of cancer [116-118]. Production of IGF-1 can increase via endocrine, paracrine and autocrine regulation by cancer cells [119-121]. Numerous studies also identified high levels of IGF-1R expression in tumors due to mutations causing IGF-1R gene amplification or other unknown mechanisms [122]. In addition, other components of the IGF-1R system, such as IRSs and IGFBPs can be deregulated in different tumors [123-127]. However, several studies indicate inverse correlation with level of IGF-1R and cancer progression [128-131]. For instance, it was reported that the level of IGF-1R is decreased particularly at late stage of prostate cancer development [128-131]. The precise reason behind this controversy remains to be elucidated.

Up-regulation of components of the IGF-1R axis induces tumor development through increased

cell proliferation, anti-apoptosis, transformation, migration and invasion [111, 114, 115]. IGF-1R induces cell proliferation through the activation of the MAPK pathway downstream receptor substrates IRS-1 and Shc [132-134]. However, the same pathways are responsible for IGF-1R induced cell differentiation [135]. It has been shown that one of the factors that control the switch between IGF-1R induced proliferation and differentiation is the balance between substrates: IRS-1 predominance induces proliferation and malignant transformation, while Shc predominance induces differentiation [135].

Cells lacking IGF-1R are unable to be transformed by several oncogenes including SV40 T antigen and/or activated Ha-Ras, c-Src, EGFR and bovine papillomavirus E5 [136]. On the other hand v-Src and GTPase deficient Gα13 are able to sustain transformation in the absence of IGF-1R [137, 138]. One of the explanations is the ability of v-Src and Gα13 to bypass IGF-1R requirement by tyrosine phosphorylating the IGF-1R substrate IRS-1, directly involved in transformation, however the exact mechanism of IGF-1R induced cell transformation remains to be investigated [139].

IGF-1R protects against apoptosis via the activation of PI3K, MAPK and 14.3.3/Raf-1/Nedd4 pathways that phosphorylates BAD and inhibits its heterodimerization with anti-apoptotic proteins Bcl-2 and Bcl-Xl, which in turn inhibits pro-apoptotic proteins Bak and Bax and cytochrome C release [105, 140-142]. This prevents caspase activation and sustains mitochondrial integrity [105, 108, 140-142].

It has been shown that proliferative and anti-apototic effects of IGF-1R require the tyrosine kinase domain with either juxtamembrane or C-terminal domains, while cell differentiation and transformation require all three domains of the receptor [137, 143, 144].

The IGF-1R system regulates cell motility and invasiveness through PI3K mediated up-regulation of p125 focal adhesion kinase (p125Fak), p130 Crk-associated substrate (p130Cas), paxillin and matrix metalloproteinases 2 and 9 (MMP-2 and 9) [145-153]. However, dual effects of IGF-1R on MMP production have been reported – stimulatory in the presence of an activated PI3K pathway and inhibitory in conditions with predominantly activated MAPK pathway [154]. Another way of increasing tumor metastasis is IGF-1 and IGF-2 induced angiogenesis through activation of hypoxia-inducible factor 1α (HIF-1α) and transactivation of other growth factors such as VEGF [155-157]. It was also demonstrated that IGF-1 can activate urokinase plasminogen activator (uPA)/uPA receptor system (uPAR) thus leading to tumor invasion [158]. Thus the IGF-1R system can regulate tumor invasion and metastasis at multiple levels [159, 160].

IGF-1R as an RTK/GPCR hybrid

It has been increasingly recognized during the last decade that despite being a prototypical RTK, IGF-1R also shares all functional characteristics of G-protein-coupled receptors (GPCRs) [88]. IGF-1R was shown to utilize G-protein signaling, G protein receptor kinase (GRK)/β-arrestin induced desensitization, receptor ubiquitination, trafficking and signaling as well as the concept of biased signaling of GPCRs [88].

GPCRs

GPCRs are the largest family of cell surface receptors, and share a similar seven-transmembranous structure and mechanism of activation [161]. In basal conditions, a GPCR is located adjacent to heterotrimeric G proteins that consist of Gα-GDP, Gβ and Gγ subunits [162]. A ligand bound receptor undergoes conformational changes to activate its GEF function followed by association with the Gα-GDP subunit and exchange of its GDP to GTP [163, 164]. This leads to dissociation of Gα-GTP from the Gβγ dimer, its interaction with second messengers and activation of the receptor signaling [163]. Intrinsic GTPase activity of Gα-subunit causes hydrolysis of GTP to GDP returning the subunit to its inactive state [165]. Gβγ triggers activation of GRKs, which phosphorylate the C-terminus of the receptor, recruiting β-arrestins [166]. β-arrestin binding uncouples the receptor from the Gα subunit leading to degradation of second messengers and further signal cessation [167]. However, there are also alternative models suggesting that certain GPCR/G protein complexes pre-exist and persist, but rearrange after receptor activation [168-174].

GPCRs: G protein

According to the classical model, when the ligand binds to a GPCR triggering dissociation of activated Gα-GTP from Gβγ subunits, both partners further activate their specific effector enzymes [175, 176]. There are four different G proteins based on their distinct α subunit: $G\alpha_s\beta\gamma$, $G\alpha_{i/o}\beta\gamma$, $G\alpha_{q/11}\beta\gamma$ and $G\alpha_{12/13}\beta\gamma$ [177, 178]. G protein specificity of GPCRs plays an important role in determination of the proper signaling downstream of the receptor [179-181]. Table 1 illustrates the involvement of different G-protein families in various GPCRs.

Gα family	Signal transduction	GPCRs	References
$G\alpha_s$ family	Stimulation of adenylyl cyclase	β1AR, β2AR, V2R	[177, 178, 182]
$G\alpha_i$ family	Inhibition of adenylyl cyclase and regulation of ion channels and activation of phosphodiesterase 6	α2AR, β1AR, β2AR, PAR1	[177, 178, 183, 184]
$G\alpha_q$ family	Activation of phospholipase C	α1AR, AT1R, PAR1, PAR2	[177, 185-187]
$G\alpha_{12/13}$ family	Activation of Rho GTPases	PAR1, PAR2	[177, 178, 188]

Table 1. *G-protein families, their signal transduction pathways and examples of coupled GPCRs.*

Some GPCRs interact with several classes of Gα proteins activating different signaling pathways [189, 190].

Additionally Gβγ utilizes five different Gβ subtypes and eleven Gγ subtypes that also regulate effectors such as adenylyl cyclase, ion channels, PI3K, phospholipase C [175, 191]. Dissociated Gβγ is also isoprenylated interacting with the plasma membrane and recruiting GRKs close to the activated receptor [192, 193].

GPCRs: GRKs

GRKs are receptor associated serine/threonine kinases that phosphorylate serine or threonine residues within the C-terminus of the activated GPCRs, thus reducing G protein mediated signaling [194]. There are seven GRK isoforms divided into three groups: the visual GRK1 and GRK7 subfamily, the GRK2 and GRK3 subfamily, and the GRK4, GRK5 and GRK6 subfamily [195-197]. Visual GRKs are expressed only in the retina, GRK4 is expressed only in the testis while all the other GRKs are expressed ubiquitously [195]. GRK isoforms differently phosphorylate GPCRs leading to distinct types of receptor signaling, trafficking and biological outcomes [195-197]. The term GRK phosphorylation "barcode" is used to describe different GRK-dependent phosphorylation patterns of GPCRs [195]. The fact that GRK2 KO mice (-/-) die at the embryonic stage while GRK3 and GRK5 KO mice are viable confirms non-identical roles of GRK isoforms [198, 199]. It has been shown that GRK2 and 3 play major roles in desensitization of β-2 adrenergic receptor (β2AR) and angiotensin II type 1 receptor (AT1R), while GRK 5 and 6 are involved in their β-arrestin mediated signaling [200-204]. However, it was also demonstrated that GRK 2 predominantly mediates β2AR desensitization in cardiac tissue, while in uterine smooth muscle this function carried out by GRK 6 [205]. Thus GPCR/GRK interaction differs depending on class of the receptor, biological function and organ specificity [195-197].

GPCRs: β-arrestins

Arrestins were firstly discovered as proteins that together with GRKs attenuate receptor signaling, a process called receptor *desensitization* [199]. There are four different arrestin isoforms: arrestins 1 and 4 regulate photoreceptors and are expressed predominantly in the retina, and arrestin 2 and 3 (called β-arrestin 1 and 2) regulate most GPCRs and are expressed ubiquitously [206]. Both β-arrestins are elongated molecules with two domains: N-domain and C-domain with a connecting C-terminal tail [206]. A polar core between the two domains is mainly responsible for β-arrestin binding to the phosphorylated receptor, though both N- and C-domain contain receptor-binding elements [207-209]. β-arrestin isoforms have identical N-terminus and different C-terminus, which promotes differences in receptor binding [210]. Both β-arrestin 1 KO (βarr1-/-) and β-arrestin 2 KO (βarr2-/-) mice survive while double KO mice (βarr1-/- and βarr2-/-) show neonatal lethality, confirming that at least one isoform is important for the vital function of β-arrestins during development [211]. Thus β-arrestins have distinct as well as overlapping biological functions and are able to replace each other in specific conditions [211].

Although firstly discovered as proteins mediating receptor desensitization, β-arrestins are now recognized as mediators of two other important functions of the receptor: i) in targeting a receptor to endocytic machinery followed by its internalization and degradation or recycling called *receptor trafficking*; and ii) activation of alternative G-protein independent receptor signaling called *β-arrestin (or β-arrestin-dependent) signaling* [210].

GPCR desensitization

β-arrestins are presumably "phosphosensors" that bind to activated and phosphorylated receptors and induce post-translational changes to the receptor [212]. The altered receptor uncouples an activated Gα subunit, which leads to signal cessation and receptor endocytosis [212]. In addition to that, β-arrestins can recruit phosphodiesterase (PDE) enzymes and induce degradation of Gα-GTP activated second messengers [167]. However, it was demonstrated recently that certain receptors can couple to G proteins even after β-arrestin binding resulting in formation of the receptor/β-arrestin/G protein complex, giving rise to sustained G protein signaling [172, 173].

β-arrestin 1 KO mice (-/-) survive but exhibit decreased β2AR signal cessation, suggesting that β-arrestin 1 plays an important role in β2AR desensitization [213]. However, it was shown that *in vitro* silencing of β-arrestin 2 affects β2AR desensitization even more than silencing of β-arrestin 1 [214]. Testing β2AR signaling in mouse embryo fibroblasts (MEFs) lacking either or both β-arrestins indicated that the role of isoforms in β2AR desensitization is interchangeable [199]. It has been identified that both β-arrestin isoforms are equally involved in desensitization of AT1R [215]. Another example is protease-activated receptors 1 and 2 (PAR1, PAR2) desensitized via both β-arrestins, with β-arrestin 1 initiating the process and thus playing predominant role [216-223].

GPCR trafficking

Receptor trafficking is another key process that controls the duration of its signaling [224-226]. β-arrestins bound to the phosphorylated receptor are able to interact with elements of the endocytic machinery such as clathrin and AP2 resulting in receptor internalization [224, 227, 228]. An internalized receptor is dephosphorylated and either recycled back to the cell surface or degraded by proteasome and lysosomes depending on the strength of the receptor/β-arrestin binding [224-229]. Two classes of GPCRs have been identified based on the strength of this interaction [219, 227]. Class A receptors preferentially and transiently bind to β-arrestin 2 followed by fast dissociation of the complex and internalization of the receptor through clathrin-coated pits [219, 227]. This class of receptor includes β2AR, α1 adrenergic (α1AR), μ opioid (MOR) and dopamine 1A (D1AR) receptors [219, 227]. Class B receptors stably bind to both β-arrestin isoforms followed by internalization of the receptor together with β-arrestins [219, 227]. This group of receptors includes AT1R, neurotensin 1 (N1) and vasopressin (V2) receptors [219, 227]. Class A receptors preferentially induce receptor recycling while class B receptors induce receptor degradation [219, 227]. The switch of C-terminal tail of the receptor changes their class affinity, indicating that the C-terminus determines class specificity [219].

GPCRs and β-arrestin signaling

The third consequence of β-arrestin binding to GPCRs is activation of G-protein independent β-arrestin signaling through interaction of β-arrestins with a variety of cellular molecules [210]. Receptor bound β-arrestins can scaffold multiple signaling molecules thus transducing signals to the MAPK, PI3K, NF-κB, p53 and other pathways [210, 224, 230]. This results in a variety of biological outcomes such as cell proliferation, anti-apoptosis, migration and invasion [230]. Interestingly the same pathway activated through G-protein or β-arrestin signaling can lead to

distinct biological outcomes [231]. For instance, β-arrestin activated ERK1/2 is mostly localized in the cytoplasm and interacts with cytosolic substrates, whereas G-protein activated ERK1/2 moves to the nucleus and acts as a transcription factor [232, 233]. Thus it is not surprising that β-arrestin activated ERK1/2 regulates proteins involved in cell motility and chemotaxis, rather than transcription activity and subsequent cell proliferation [234].

Receptor class specificity also influences the length of β-arrestin induced signaling: transient interaction of Class A receptors with β-arrestin 2 activates short β-arrestin signaling, while stable interaction of Class B receptors with both β-arrestins leads to a stable β-arrestin signaling [224]. However, there are exceptions from the classical pattern of β-arrestin mediated receptor trafficking and signaling. It has been demonstrated that PAR2 receptor is degraded faster through interaction with β-arrestin 1, while β-arrestin 2/PAR2 interaction leads to more stable association and signaling [223].

In addition regardless of the class specificity β-arrestin isoforms can play co-dependent or reciprocal role in regulation of the β-arrestin induced signaling [218, 224]. Furthermore reciprocal regulation can appear as a positive input of β-arrestin 1 and negative by β-arrestin 2 or vice versa [218].

Distinct roles of β-arrestin isoforms in receptor desensitization, endocytosis and signaling are partially explained by recently identified specific conformations of β-arrestin/GPCR interaction. It has been shown that interaction of β-arrestins with the C-terminus of GPCRs mediate receptor internalization and signaling, while additional interaction of β-arrestins with the receptor transmembrane core mediates desensitization [172, 173]. However, β-arrestins/GPCR interactions that discriminate the roles of isoforms in receptor endocytosis from signaling remain to be investigated.

Other functions of β-arrestins

It is important to mention that β-arrestin isoforms also specifically regulate functions other than desensitization, trafficking and β-arrestin signaling of the receptor [235, 236]. β-arrestin 1 can move to the nucleus in response to receptor activation, enhancing histone acetylation and gene transcription, while β-arrestin 2 is involved in nuclear export of Mdm2 and JNK3 kinases [237-240]. It has been suggested that these functions are controlled by non-identical C-terminal regions of the isoforms [237-240].

Posttranslational modifications of β-arrestins

Various posttranslational modifications of β-arrestins such as ubiquitination and phosphorylation regulate GPCR functions [227, 241]. Ubiquitination pattern of β-arrestins correlates with GPCR class specificity: transient β-arrestin ubiquitination with class A and persistent β-arrestin ubiquitination with class B [227, 242]. The state of β-arrestin phosphorylation defines receptor endocytosis for example, dephosphorylation of serine residue 412 of β-arrestin 1 increases its affinity for clathrins [243-245]. Oligomerization state also controls β-arrestins activity [246]. β-arrestin 1 or 2 monomers are ubiquitinated by Mdm2 and involved in receptor desensitization as well as transcriptional activity of β-arrestin 1 [246, 247]. However, β-arrestin 2 oligomers are involved in the nuclear export of Mdm2 [246-248].

Different models of GPCR regulation by β-arrestin 1 and 2 isoforms

The β2AR belongs to the class A receptors and its C-terminus phosphorylation leads to the transient binding of β-arrestin 2 followed by receptor internalization and recycling [219]. However, both β-arrestin isoforms can induce arrestin dependent ERK activation of β2AR [204]. On the other hand, AT1R belongs to class B receptors and its C-tail phosphorylation recruits both β-arrestins that stably bind to the receptor [219]. However, the β-arrestin isoforms play a reciprocal role in activation of signaling: β-arrestin 1 induces, while β-arrestin 2 inhibits the arrestin signaling of AT1R [217]. Distinct roles of β-arrestin isoforms in endocytosis and signaling of different GPCRs are shown in Table 2.

GPCR	Desensitization	Traffiking	Signaling	References
β2AR	β-arr2>β-arr1	β-arr2>β-arr1	β-arr1↑/β-arr2↑	[204, 218, 219]
AT1R	β-arr1/β-arr2	β-arr1/β-arr2	β-arr1↓/β-arr2↑	[218, 219, 232]
PAR1	β-arr1>β-arr2	Not essential	β-arr1↑/β-arr2↓	[216-220]
PAR2	β-arr1>β-arr2	β-arr1/β-arr2	β-arr1↑/β-arr2↑	[218, 219, 221-223]

Table 2. *Role of β-arrestin isoforms in GPCRs desensitization, endocytosis and signaling. ↑- stimulatory role; ↓- inhibitory role; > - more potent regulation.*

Simplified models of G-protein, GRK and β-arrestin isoform interactions with the most extensively studied β2AR and AT1R GPCRs are illustrated in Figure 2.

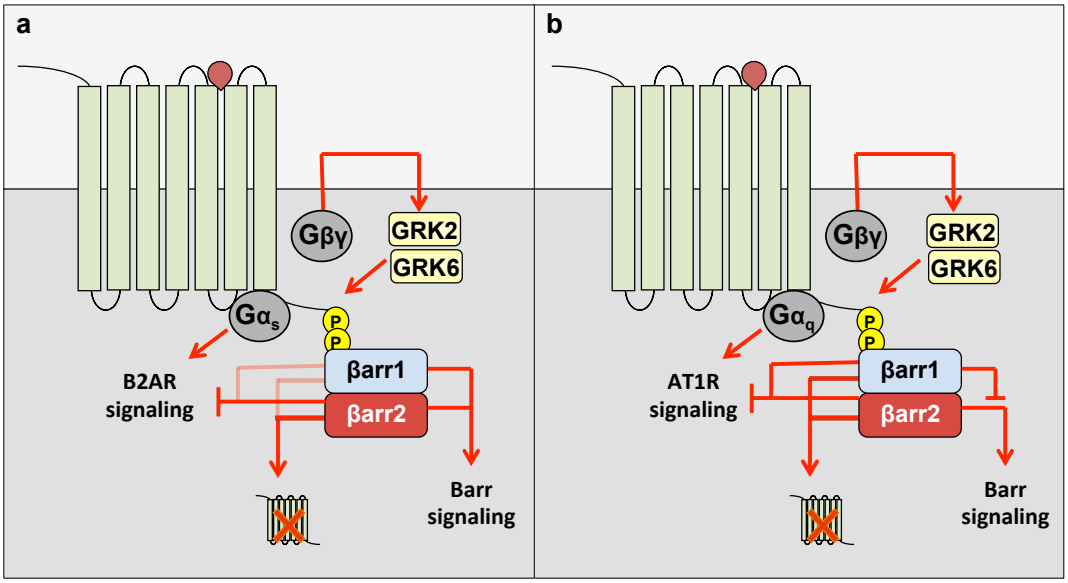

Figure 2. *Schematic illustration of the roles of G proteins, GRKs and β-arrestins in β2AR and AT1R signaling. a) β2AR activation is followed by interaction with $G\alpha_s\beta\gamma$, phosphorylation by GRK2/6 and β-arrestin1/2 binding; b) AT1R activation followed by interaction with $G\alpha_q\beta\gamma$, phosphorylation by GRK2/6 and β-arrestin1/2 binding. Distinct effects of β-arrestins on desensitization, downregulation and signaling are shown with red lines. Arrowed lines indicate stimulatory and blunted lines - inhibitory effects. Transparent lines indicate weaker effects.*

GPCRs: biased signaling

Since the discovery of multiple pathways activating downstream of the stimulated receptor, the term biased agonism was introduced [249]. The concept of biased agonism expanded particularly after the discovery of β-arrestin induced signaling of GPCRs [226, 250]. This concept defines the ability of the ligand to induce and/or receptor to acquire conformation, causing activation of only specific pathways downstream of that receptor [189, 190, 251, 252]. Various ligands have been described that specifically inhibit the G protein while activating the β-arrestin mediated signaling pathways or vice versa [253]. The possibility to activate specific pathways could be widely applied in the drug discovery field in order to minimize side effects [254].

IGF-1R as a GPCR

IGF-1R: G protein

It has been demonstrated that various RTKs including the IR, IGF-1R, EGFR, PDGFR and TrkA can utilize G-proteins for the activation of their signaling [255-261].

It was shown that IGF-1R also couples to $G\alpha_i$ subunit activating MAPK pathway that can be blocked by compounds inhibiting G-proteins [262, 263]. In a basal state, $G\alpha_i$ and $G\beta\gamma$ both associate with IGF-1R [264]. IGF-1 stimulation releases $G\beta\gamma$ subunit but increases the association of IGF-1R with the $G\alpha_i$ subunit, thus also utilizing G proteins signaling [264]. Importantly, IGF-1R induced G-protein activation can result from direct IGF-1R/G-protein interaction as well as through transactivation of GPCRs [262, 264-266].

IGF-1R: GRKs

Involvement of GRKs in desensitization of various RTKs was also reported [258, 259]. Activation of EGFR, PDGFR and TrkA were shown to translocate GRK2 to the plasma membrane resulting in activation of MAPK pathway [258, 267, 268].

Activated IGF-1R recruits GRKs that phosphorylate serine residues at its C-terminus [269]. There is a contrasting effect between GRK2 and GRK6 on IGF-1R function [269]. Both GRKs interact with IGF-1R, however GRK2 phosphorylates IGF-1R serine residue 1248 and induces transient β-arrestin 1/IGF-1R binding, while GRK6 phosphorylates serine residue 1291, inducing stable interaction of β-arrestin 1 with IGF-1R [269].

IGF-1R: β-arrestins

Both β-arrestin isoforms were shown to interact with the IGF-1R [264, 270, 271]. It has also been demonstrated that β-arrestin 1 interacts with the C-terminus of IGF-1R transiently binding to serine residue 1248 and stably binding to serine residue 1291 whereas the site of β-arrestin 2 interactions with IGF-1R has not yet been described [272, 273].

IGF-1R tyrosine kinase inactivation ("desensitization")

One described mechanism of termination of IGF-1R TK signaling is the dissociation of IRS-1 from the receptor followed by Mdm2 dependent ubiquitination and degradation of IRS-1 [274].

It was shown that β-arrestin 1 competes with IRS-1 for Mdm2 binding and IGF-1/insulin treatment downregulate β-arrestin 1 thus enhancing IRS-1/2 degradation and signal cessation [274-278]. On the other hand, β-arrestin 1 is directly involved in IGF-1R degradation thus also contributes to the TK signaling cessation [272].

IGF-1R trafficking

The role of the β-arrestin system in the downregulation of IGF-1R and other RTKs such as EGFR, PDGFR and IGF-1R has been reported [257, 272, 279]. β-arrestin 1 is recruited to the EGFR upon ligand stimulation, inducing receptor endocytosis [264, 277, 279]. Residues 319-418 of the C terminus of β-arrestin 1 are involved in EGFR/β-arrestin 1 interaction with clathrin, indicating the importance of β-arrestin 1 dephosphorylation [264, 277, 279]. β-arrestin 1 also mediates interaction of PDGFβ with the GPCR EDG1 leading to internalization of PDGFR/EDG1 complex through clathrin-coated pits [257, 280]. Ligand binding to TrkA also recruits β-arrestin 1 to the receptor leading to the clathrin mediated receptor endocytosis [258, 281]. It has been suggested that TrkA/β-arrestin 1 association is also mediated through GPCR EDG2 [258, 281]. However, the role of the β-arrestin 2 isoform in RTK internalization and signaling is mostly unexplored.

It is demonstrated that clathrin dependent internalization of IGF-1R is mediated by both β-arrestin isoforms [270]. Dephosphorylation of C-terminal serine 412 residue of β-arrestin 1 is a prerequisite for this internalization [270]. The ubiquitin ligase Mdm2 is recruited to activated IGF-1R through β-arrestin 1 leading to receptor degradation [273, 282]. Both β-arrestin isoforms were shown to induce IGF-1R ubiquitination and downregulation with β-arrestin 1 being more potent than β-arrestin 2 [272]. The mechanism and biological outcomes of this difference have not yet been investigated.

IGF-1R and β-arrestin induced signaling (TK independent signaling)

Despite indication of β-arrestin 1 involvement in ligand-induced EGFR downregulation, no studies have shown the effect of β-arrestins on EGFR mediated MAPK signaling [264, 277, 279, 283]. However, it has been shown that PGE2 induced EGFR transactivation followed by Akt signaling is mediated through β-arrestin 1 [284]. It has been demonstrated that ligand binding to TrkA recruits β-arrestin 1 thus mediating activation of MAPK signaling [281]. The specific role of β-arrestin isoforms on PDGF and FGFR signaling remains unknown [257, 280].

Ligand dependent interaction of IR with β-arrestin 2 was shown to activate Akt through Src thus contributing to insulin PI3K signaling [285]. β-arrestin 1 was not shown to affect insulin induced metabolic or mitogenic signaling pathways of IR [264, 274-277].

Recruitment of β-arrestin 1 to IGF-1R has been shown to mediate IGF-1 induced activation of MAPK and PI3K signaling [264, 270, 271, 286]. Activation of MAPK requires β-arrestin 1 dephosphorylation, its association with clathrin as well as recruitment of Mdm2 [270, 282]. The mechanism of IGF-1R/β-arrestin 1 mediated PI3K activation is not fully investigated [286].

IGF-1R: biased signaling

Since the discovery of the GPCR properties of RTKs, the paradigm of biased agonism was also introduced to the field of RTKs [287-289].

Different extracellular domains of RTKs responsible for activation of distinct signaling pathways have been identified [290-296]. Various alterations of ligands, presence of co-receptors and certain intracellular proteins were shown to induce allosteric modulations of RTKs [297-301]. For instance, PKCε was shown to interact with the cytosolic domain of EGFR inhibiting PLCγ but not ERK and Akt signaling and altering sensitivity to EGFR inhibitors [297, 298]. All these studies offer new possibilities to target RTKs with various biased agonists for better therapeutic outcomes [292-296].

A limited number of biased agonists have been described for RTKs [287]. It has been demonstrated that a specific inhibitor of FGFR, SSR128129E, binds to the extracellular domain of the receptor changing its conformation and inhibiting MAPK but not phospholipase C activation [302, 303]. A compound interacting with the juxtamembrane domain of TrkA was shown to inhibit receptor inducing its dimerization and biased signaling [304].

Biased signaling of IGF-1R was first described when the kinase inhibitor cyclolignan picropodophyllin (PPP) was shown to induce IGF-1R downregulation and biased ERK signaling [305]. Similarly, the antimicrobial peptide LL-37 has been demonstrated to induce IGF-1R degradation with activation of biased signaling [306]. The IGF-1R antibody figitumumab (CP-751871 or CP), previously described as a receptor antagonist also maintains agonist like properties, it induces IGF-1R downregulation and activates β-arrestin 1-dependent ERK signaling [307]. Since the discovery of these properties, it becomes important to test the effect of all IGF-1R targeting molecules on all pathways downstream of IGF-1R.

To sum up, all main characteristics of GPCR activation and desensitization were detected at the IGF-1R: 1) IGF-1 binding activates the G protein signaling cascade, as well as the classical tyrosine kinase signaling [264]; 2) IGF-1R phosphorylation by GRK2 or GRK6 at C-terminal serine residues allows β-arrestin binding to the receptor [269]; 3) the GRK phosphorylated receptor undergoes ubiquitination and degradation or recycling to the cell surface [269, 272]; 4) β-arrestin recruitment leads to cessation of IGF-1R signaling [272]; 5) β-arrestin binding activates a second wave of IGF-1R signaling called β-arrestin dependent signaling [282]; 6) IGF-1R targeting molecules can be classified based on their biased agonistic properties [88]. Thus it has been proposed to classify the IGF-1R as a functional hybrid of both RTK and GPCR [88].

Simplified overview of the previously known roles for β-arrestins in IGF-1R activation and signaling is provided in Figure 3.

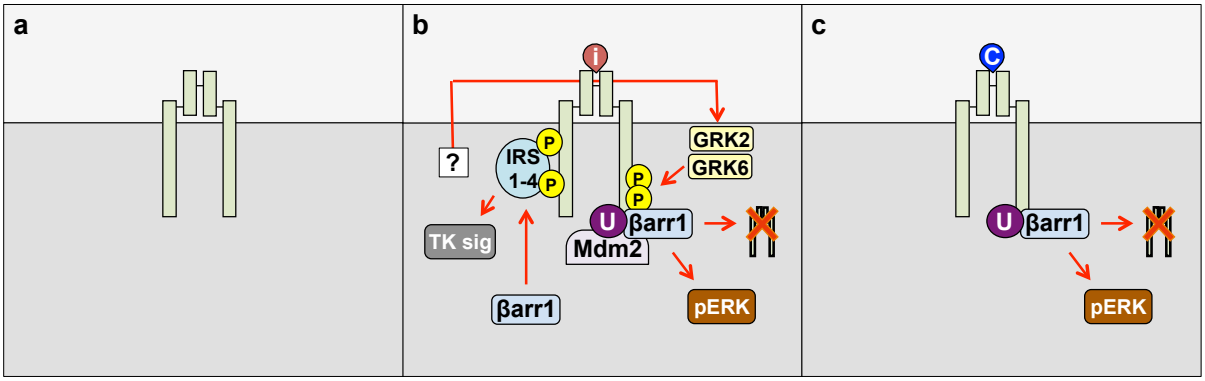

Figure 3. *a) Absence of the ligand determines absence of the TK activity as well as IGF-1R/β-arrestin interaction and β-arrestin signaling; b) IGF-1 binding leads to TK activation, followed by GRK phosphorylation and recruitment of β-arrestin 1 and Mdm2 leading to receptor degradation and ERK signaling; c) CP binding inhibits the TK activity of the receptor but recruits β-arrestin 1 leading to receptor degradation and ERK signaling.*

β-arrestins and cancer

β-arrestin signaling of GPCRs and RTKs regulates multiple cancer inducing cellular functions such as cell proliferation, migration, invasion, anti-apoptotic signaling and cell cycle progression [218, 222, 224, 226, 234, 236, 284, 308-314]. β-arrestins activate these functions by scaffolding various cytosolic proteins as well as directly regulating expression of genes [218, 224, 226, 234, 237, 315, 316]. Both β-arrestins are involved in different cancer related receptor functions [236, 314].

Overexpression of β-arrestin 1 in transgenic mice increases VEGF induced MMP-9 activity and cell growth [317]. Stimulation of various GPCRs (FPR1, AT1R, V2R, and CXCR2) induces apoptosis in β-arrestins 1 and 2 knockout mouse embryonic fibroblasts (MEFs) while expression of β-arrestins rescues this effect [309].

β-arrestins mediate neurokinin-1 receptor (NK1R) induced ERK activation through Src, leading to cell proliferation and anti-apoptosis [318]. IGF-1 activation causes a β-arrestin 1 mediated increase of pERK1/2 and pAkt leading to cell proliferation and anti-apoptosis [282, 286]. The C-terminal domain of β-arrestin 2 plays specific role in anti-apoptotic signaling through adaptor protein AP2 [319, 320]. β-arrestin 2 mediates AT1R induced anti-apoptotic signaling through MAPK and PI3K pathway by phosphorylation of apoptotic protein BAD [321]. β-arrestin 2 also attenuates GSK-3β apoptotic signaling of Toll-like receptor 4 [322]. Similarly, β-arrestin 2 inhibits resveratrol induced GSK-3β mediated apoptosis of endometrial cancer cells [323]. CXCR4 induced cell migration is mediated by β-arrestin 2 activated MAPK pathways [324, 325]. However, β2AR induced dephosphorylation of β-arrestin 2 promotes apoptosis after UV exposure via stabilization of IκBα/NF-κB association [326]. In addition, activated δ opioid receptor (DOR) recruits Mdm2 through β-arrestin 2 thus inhibiting Mdm2/p53 interaction and increasing p53-mediated apoptosis [327].

Specific involvement of β-arrestin isoforms in various types of cancer summarized in Table 3.

Cancer	Isoform	Mechanism of involvement	Ref.
Colon cancer	β-arrestin 1	Mediates PGE2 induced EGFR transactivation	[284]
	β-arrestin 2	Mediates Wnt signaling	[313]
Bladder cancer	β-arrestin 1	Not involved in TPβR induced malignancy	[328]
	β-arrestin 2	Mediates TPβR signaling	[329]
Blood cancers	β-arrestin 1	Elevated in ALL, inhibits Notch1 signaling	[330]
	β-arrestin 2	Involved in CML, mediates Wnt signaling	[310]
Prostate cancer	β-arrestin 1	Mediates Wnt signaling	[331]
	β-arrestin 2	Downregulates AR and TβRIII; Mediates β2AR induced c-Src activation	[332-335]
Pancreatic cancer	β-arrestin 1	N/D	
	β-arrestin 2	Mediates CXCR4/7 induced MAPK signaling; Possibly mediates increased Shh signaling	[336-339]
Brain tumors	β-arrestin 1	Low phospho-S412 correlated with low survival in GBM	[340]
	β-arrestin 2	Possibly mediates increased CXCR4 signaling in MBM	[341]
NSCLC	β-arrestin 1	Mediates PGE2 induced EGFR transactivation and nAChRs signaling; Nuclear increase correlates with poor prognosis	[308, 342, 343]
	β-arrestin 2	Decrease correlates with poor prognosis	[312]
Breast cancer	β-arrestin 1	Mediate LPAR and PAR2 signaling and HIF-1α dependent activation of VEGF-A; Decreased with tumor progression, correlating with poor prognosis	[222, 344-346]
	β-arrestin 2	Mediate LPAR, PAR2 and KISS1R signaling; TβRIII dependent inhibition of NF-κB; Possibly mediates CXCR4 signaling; Increased with tumor progression, correlating with poor prognosis	[222, 344, 346-349]
Ovarian cancer	β-arrestin 1	Mediates ETAR signaling and EGFR transactivation	[311]
	β-arrestin 2	Mediates ETAR signaling and EGFR transactivation	[311]

Table 3. *Mechanisms of β-arrestin 1 and 2 involvement in various types of cancer.*

1.4. Tumor suppressor p53 pathway

The p53 pathway is a crucial tumor suppressor pathway that is probably inactivated in all tumors [18, 350-352]. p53 prevents cancer by maintaining genetic stability in damaged cells and eliminating incipient tumor cells [353, 354]. Oncogenic stress induces p53 phosphorylation and acetylation, which inhibits its interaction with the Mdm2 ubiquitin ligase and thereby stabilizes p53, and activates p53 as a transcription factor [353-357]. Activated p53 transcriptionally activates target genes and induces growth arrest and if necessary, apoptosis or senescence [353-356]. In an unstressed environment p53 is degraded and kept at low levels by

Mdm2 that binds to p53, mono-ubiquitinates it and exports to the cytosol where p53 is degraded in the proteasome [353-356]. Expression of Mdm2 is also transcriptionally regulated by p53 through a negative feedback loop [353-357].

The TP53 gene is mutated in around 50% of cancers [358, 359]. Most TP53 mutations are missense mutations that disrupt p53 DNA binding [358, 359]. Mutant p53 can exert a dominant negative effect on a wild type p53 [358, 359]. Another mechanism of p53 inactivation is overexpression of its natural inhibitor, the E3 ubiquitin ligase Mdm2, in cancer cells [358-361].

1.5. Link between IGF-1R signaling and p53 pathway

There is a solid amount of data indicating that p53 interacts with the IGF-1 pathway 1) at the transcriptional level; 2) through regulation of metabolic processes; 3) and through convergence of the IGF-1R and p53 signaling pathways [362, 363].

Link at the transcriptional level

It has been shown that wild type p53 suppresses transcription of IGF-1R, possibly as part of its apoptotic effect, however mutant p53 activates IGF-1R expression [364-366]. Wild type p53 also inhibits transcription of IGF-2 and induces transcription of the IGFBP2 and IGFBP3 genes [367-369]. In addition, p53 homologs p63 and p73 proteins also inhibit IGF-1R transcription while their mutant forms fail to do so [370]. Other tumor suppressors such as BRCA1, pVHL and WT1 were also demonstrated to reduce expression of IGF-1R [371-374].

Regulation of metabolic processes

The interplay between IGF-1R and the p53 pathway is also implicated in regulation of metabolic pathways [375]. IGF-1 directly regulates glucose uptake and inhibits hepatic production of glucose [376]. However, wild type p53 inhibits glycolysis through repression of *GLUT1* and *GLUT4* genes, while mutant p53 lacks this function [377]. Wild type p53 also promotes hepatic production of glucose thus possibly preventing glycolysis utilized by cancer cells [378]. Another example is that IGF-1 stimulates expression of the transcription factor SREBP involved in sterol synthesis, while p53 inhibits SREBP and thus lipogenesis [379, 380].

Convergence of signaling pathways

Mdm2 is a well-described ubiquitin ligase for both p53 and IGF-1R [273, 381]. It was demonstrated that IGF-1 signaling affects the activity of Mdm2 towards p53 [382, 383]. IGF-1R activation induces PI3K pathway leading to Akt translocation to the nucleus followed by up-regulation of transcription factors involved in cell growth and anti-apoptosis [382, 383]. However, Akt also phosphorylates Mdm2 inhibiting its association with predominantly nuclear protein ARF [382-384]. This leads to nuclear export of the Mdm2/p53 complex followed by cytoplasmic proteasomal degradation of p53 [383-386]. p53 can inhibit the PI3K pathway through activation of the inhibitor of this pathway, PTEN [387].

It was also shown that IGF-1 induces Mdm2-dependent p53 degradation in response to DNA damage through activation of MAPK pathway [384, 388-390]. In addition p53 can inhibit MAPK pathway through caspase-mediated cleavage of the ERK2 [391].

β-arrestin 1 was shown to mediate Mdm-2 dependent IGF-1R degradation and MAPK signaling [272, 282]. Interestingly β-arrestin 1 also facilitates Akt induced p53 degradation by Mdm2 downstream of β2AR [392]. However, the role of β-arrestins in IGF-1 induced Mdm2/p53 interaction has not been elucidated.

Thus IGF-1R and p53 pathway are interconnected at multiple levels and dissection of this complex interplay has a major impact on understanding of basic physiology as well as on improvement of specific anti-cancer therapies.

A simplified scheme of IGF-1R interplay with the p53 pathway is provided in Figure 4.

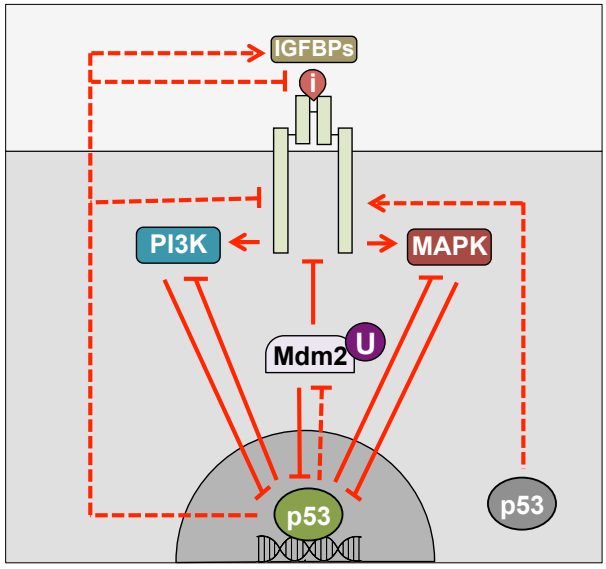

Figure 4. *Schematic illustration of interplay between IGF-1R and wild type (green) and mutant (grey) p53. Dashed line indicates transcriptional regulation, solid line indicates regulation at the protein level. Arrowed lines indicate stimulatory and blunted lines indicate inhibitory effects.*

2. AIM OF THE THESIS

The main aim of this thesis was to investigate in detail the IGF-1R/β-arrestin/Mdm2/p53 axis in cancer and to explore the potential use of its components as therapeutic targets.

Paper I: To analyze the molecular interplay between p53 and IGF-1R pathways through Mdm2.

Paper II: To investigate the role of β-arrestin isoforms in the IGF-1R/β-arrestin/Mdm2/p53 axis.

Paper III: To reveal a potential of co-targeting the IGF-1R/β-arrestin/Mdm2/p53 axis with the MAPK pathway.

Paper IV: To reveal the potential of co-targeting the IGF-1R/β-arrestin/Mdm2/p53 axis with DNA damage inducing drugs.

3. RESULTS AND CONCLUSIONS

3.1. Paper I: Unbalancing p53/Mdm2/IGF-1R axis by Mdm2 activation restrains the IGF-1-dependent invasive phenotype of skin melanoma

Background and rationale:

p53 mutations are rare in melanoma and instead p53 is often inhibited by up-regulation of the ubiquitin ligase Mdm2 [393-396]. Thus, the disruption of the Mdm2/p53 interaction could be a rational approach for melanoma treatment. The IGF-1R is another important player in melanoma cell survival and is also regulated by ubiquitin ligase Mdm2 [273, 397, 398]. As both p53 and IGF-1R are substrates of Mdm2, we investigated the impact of Mdm2/p53 disruption on function of IGF-1R in a melanoma model.

Results and conclusions:

We used the prototypical p53-reactivator Nutlin-3 to disrupt Mdm2/p53 complex in four melanoma cell lines, two with wild type (wtp53) and two with mutant p53 (mtp53).

Since Mdm2 is a ubiquitin ligase for IGF-1R, we first investigated the effect of Nutlin-3 on the levels of Mdm2 and IGF-1R. We demonstrated that in wtp53 cells, Nutlin-3 causes a dose and time dependent accumulation of p53 and Mdm2, accompanied by a decrease in the level of IGF-1R. No significant effects on the level of p53, Mdm2 or IGF-1R were observed in mtp53 cells. These data indicate that Nutlin-3 affects IGF-1R level following increase of Mdm2.

It has been demonstrated that Mdm2 induces IGF-1R ubiquitination followed by its degradation and pERK signaling in response to IGF-1 stimulation [272, 273, 282]. Thus, we next tested whether IGF-1 stimulation was essential for the Nutlin-3 mediated IGF-1R degradation. Cells were Nutlin-3 treated, serum starved, IGF-1 stimulated and monitored for changes in the level of IGF-1R using western blot (WB). We demonstrated that IGF-1R degradation only occurred in the presence of IGF-1, despite similar levels of p53 accumulation in both serum free and IGF-1 conditions. This effect was also obvious only in wtp53 cells.

We next investigated the mechanism of Nutlin-3 dependent IGF-1R downregulation. Using qPCR we excluded transcriptional origin of Nutlin-3 mediated IGF-1R decrease. Using immunoprecipitation (IP) we investigated whether IGF-1R is physically associated with and ubiquitinated by Mdm2 after Nutlin-3 treatment. In serum starved conditions recruitment of Mdm2 to the IGF-1R was very low, increasing after Nutlin-3 treatment or IGF-1 stimulation and reaching maximum after Nutlin-3 treatment followed by IGF-1 stimulation. A similar pattern was observed for ubiquitination level: Nutlin-3 or IGF-1 treatment alone increased IGF-1R ubiquitination, while double treatment increased it further. We next tested Nutlin-3 induced IGF-1R degradation in cells expressing low level of Mdm2 (SAOS) and full length (MEF wt) and C-terminus truncated (Δ1245) IGF-1R. In SAOS2 cells Nutlin-3 treatment did not induce either p53 or Mdm2 accumulation, while in MEF wt and Δ1245 it induced both. However, Nutlin-3 induced IGF-1R degradation was only observed in MEF wt cells. These data confirmed that Mdm2-mediated ubiquitination of the IGF-1R is a key mechanism of Nutlin-3 induced IGF-1R downregulation.

Since IGF-1R ubiquitination by Mdm2 also activates pERK we next tested the effect Nutlin-3 may have on IGF-1R signaling. We stimulated untreated and Nutlin-3 treated cells with IGF-1 for up to 60 minutes and observed an early and overall increase of pERK in Nutlin-3 treated samples, compared to untreated. This increase was eliminated by IGF-1R downregulation and was not observed in SAOS-2 and Δ1245 cells. These results demonstrated that Nutlin-3 acts as a partial agonist for IGF-1R induced pERK signaling through Mdm2.

IGF-1R is a potent inducer of cell transformation, proliferation, invasion and metastasis [117, 148, 399]. Thus, we next investigated the effect of Nutlin-3 on these biological behaviours. Using a soft agar assay, cell viability assay and FACS analysis we demonstrated that Nutlin-3 treatment inhibits colony formation, slightly increases total cell number, and decreases overall IGF-1 response. Using both monolayer wound healing and transwell chamber assays we demonstrated that Nutlin-3 increased at early stage and decreased at later time points IGF-1 induced migration, particularly in wtp53 cells. However, a matrigel invasion assay and monitoring of MMP-2 activation showed that Nutlin-3 completely inhibits IGF-1 induced invasion. To sum up, Nutlin-3 inhibits IGF-1R induced cell proliferation/survival, has a two-step effect on IGF-1 induced cell migration and completely abolishes IGF-1 induced invasion.

The main findings of the study are that 1) p53/Mdm2 disruption activates IGF-1R interaction with Mdm2; 1) Nutlin-3 comprises agonistic properties towards IGF-1R induced pERK; 2) p53/Mdm2/IGF-1R axis could be used as a potential target for anti-cancer therapy.

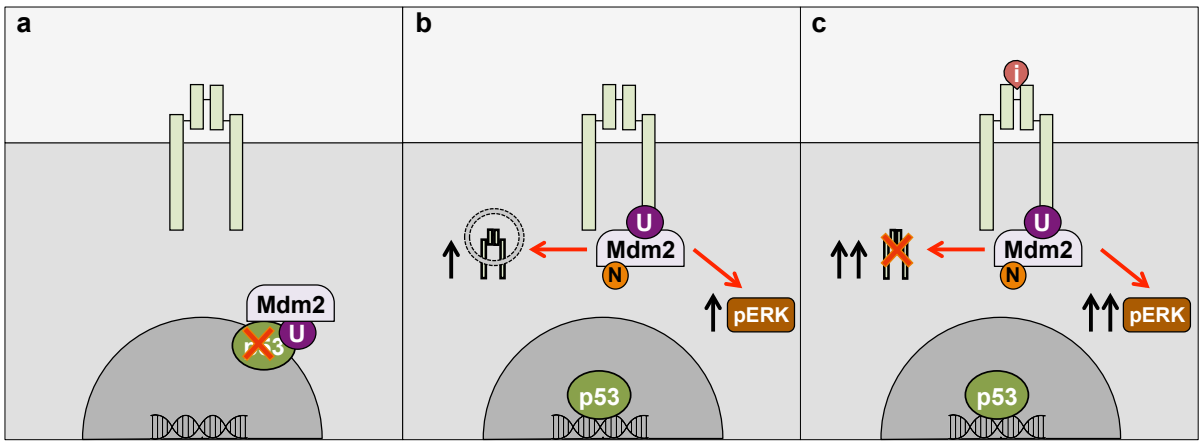

Figure 5. *Schematic illustration of the p53/Mdm2/IGF-1R axis in the a) absence of Nutlin-3 and IGF-1; b) presence of Nutlin-3; c) presence of both, Nutlin-3 and IGF-1.*

3.2. Paper II: Functional antagonism of β-arrestin isoforms balance IGF-1R expression and signaling with distinct cancer-related biological outcomes

Background and rationale:

The roles of the two β-arrestins (β-arrs) with similar structure are well characterized in various GPCRs [224, 226]. In regards to their function at the IGF-1R, it is mainly the function of β-arr1 that has been described so far. It has been demonstrated that β-arr1 induces ligand dependent IGF-1R degradation, activates late MAPK signaling, and is able to mediate resistance to IGF-1R targeting in cancer [272, 282, 305]. However, it was also shown that both β-arrs can bind and ubiquitinate the IGF-1R leading to its degradation [269, 272]. Yet little is known about the specific function of β-arr2 on IGF-1R expression and function. In this study, we particularly focused on the role of the β-arr2 isoform and identified distinct roles for the two isoforms on IGF-1R system as well as on the interconnected p53 pathway.

Results and conclusion:

It has been shown that β-arr isoforms can differently downregulate various GPCRs [219, 225, 227]. Thus, we first studied the effect of β-arr2 on IGF-1R expression. β-arr1 knock out (β1KO), β-arr2 knock out (β2KO) and wild type (WT) MEF cells were serum starved and stimulated with IGF-1 for 0, 12 and 24 h followed by monitoring of IGF-1R degradation rate by WB. Our results indicated that IGF-1R degradation is impaired in β1KO, and enhanced in β2KO cells compared to WT cells. We next transfected HEK and WT MEF cells with β-arr1 or β-arr2 encoding plasmids or siRNAs and showed that β-arr1 up-regulation and β-arr2 inhibition enhances, whilst β-arr1 inhibition and β-arr2 up-regulation impairs IGF-1R degradation. Importantly, IGF-1R level was decreased in serum starved β-arr2 up-regulated cells prior to stimulation. This experiment demonstrated that in contrast to β-arr1, β-arr2 inhibits the rate of ligand dependent degradation of IGF-1R.

It has been also demonstrated that β-arr1 sustains IGF-1 induced ERK activation [282]. Therefore, we next tested the effect of β-arr2 on modulation of IGF-1R signaling. We serum starved and stimulated β1KO, β2KO and WT MEF cells with IGF-1 for 0, 2, 5, 10, 30 and 60 minutes and indicated that ERK activation lasts longer in β2KO and shorter in β1KO compared to WT cells. Down- or upregulation of β-arr2 in HEK and MEF cells confirmed that β-arr2 inhibition prolongs and up-regulation shortens IGF-1 activated ERK. These data indicate opposing roles for β-arr1 and β-arr2 on IGF-1-induced late ERK activation.

β-arr1 is known to control IGF-1R degradation and signaling through Mdm2 dependent ubiquitination of the C-terminus of IGF-1R [282]. Thus, we next tested impact of Mdm2 on β-arr2 activity by altering β-arrs in MEF cells, expressing a C-terminus truncated IGF-1R (Δ1245), SAOS-2 cells with low endogenous and U2OS cells with high endogenous level of Mdm2. We observed a similar effect for β-arr2 only in U2OS cells, while in Δ1245 and SAOS-2 cells IGF-1R degradation and signaling was unchanged after β-arr2 alteration. This data indicated a role for Mdm2 and the C terminus of IGF-1R in the modulation of IGF-1R function by β-arr2.

We next tested whether β-arr2 also binds to and induces ubiquitination of the IGF-1R. We up-regulated IGF-1R and either β-arr1 or β-arr2 in U2OS cells, followed by serum starvation, IGF-1 stimulation and immunoprecipitation of the β-arrs. We analyzed the immunoprecipitates and demonstrated that β-arr2 preferentially binds to and induces ubiquitination of a ligand-free IGF-1R, while β-arr1 - of a ligand occupied IGF-1R.

Next, we assessed the biological outcomes of modulation of IGF-1R expression and ERK activation by β-arr isoforms. We modulated β-arr1 or 2 in U2OS and SAOS-2 cells, followed by serum starvation, IGF-1 stimulation and analysis of proliferation/survival and cell cycle distribution using PrestoBlue and FACS. We revealed increased total cell number and cell cycle progression in response to IGF-1 after β-arr1 up-regulation and β-arr2 inhibition in both cell lines. β-arr2 up-regulation and β-arr1 inhibition in contrast, reduced cell number as well as IGF-1 response causing cell cycle arrest. Interestingly, SAOS-2 were affected more and arrested in G2 phase, while U2OS cells were arrested in both G1 and G2 phases, suggesting that p53 absence facilitates a G1/S transition even without sustained proliferative signals.

We proposed that β-arr2 predominance decreases cell viability through β-arr2 mediated downregulation of IGF-1R during serum starvation and an activated p53 pathway. We confirmed both scenarios by monitoring a gradual decrease in IGF-1R level in serum free conditions and re-activation of p53 in β-arr2 up-regulated and β-arr1 inhibited cells. Thus both receptor depletion and increased p53 contributes to IGF-1 insensitivity in β-arr2 predominant conditions.

The main findings of this study are 1) opposing roles of β-arr isoforms in modulating IGF-1R function; 2) an important role of β-arr1/2 balance in cell cycle progression; 3) identification of anti-cancer therapeutic potential of β-arr1/2 system with an impact on both pro-oncogenic IGF-1R signaling and tumor suppressor p53 pathway.

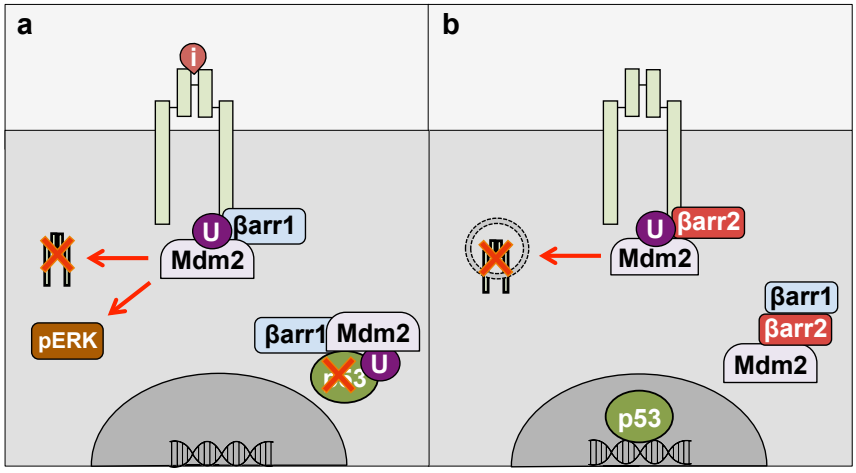

Figure 6. *Schematic illustration of prevailing manner of β-arr1 or β-arr2 interaction with p53 and IGF-1R in the a) presence of IGF-1; b) absence of IGF-1 ligand.*

3.3. Paper III: Enhanced response of melanoma cells to MEK inhibitors following unbiased IGF-1R down-regulation

Background and rationale:

Due to frequent mutation of RAF or RAS genes, inhibition of the downstream MAPK is a rational approach for melanoma treatment [400]. However, current MAPK inhibitors develop rapid resistance soon after administration that is associated with a more aggressive disease recurrence [401-403]. Melanoma cells acquire alternative routes to sustain proliferative signaling, including IGF-1R exploitation. Thus, co-targeting of MAPK inhibition with IGF-1R downregulation is a reasonable strategy for better outcome of treatment [404-406]. Yet IGF-1R downregulation can be associated with inappropriate activation of β-arr induced biased MAPK signaling [289, 305, 307, 407, 408]. In this study, we explore co-targeting of MAPK inhibition with balanced and β-arr signaling biased IGF-1R downregulation to aim to maximize drug sensitivity of melanoma cells.

Results and conclusion:

Since RAS and RAF mutations occur in high percentage in melanoma we first tested RAS/RAF status and sensitivity to MEK1/2 inhibitor U0126 in a panel of melanoma cell lines. We confirmed by sequencing that DFB and Mel28 cells contains BRAF mutation, while BE contains an NRAS mutation. Cells cultured in media with and without serum followed by WB analysis indicated that the highest level of basal pERK1/2 was in Mel28 and lowest in BE cells, which further increased with serum. We demonstrated a U0126 dose-dependent decrease in cell viability with a higher response in BRAF positive cells. We also demonstrated a higher sensitivity to U0126 in the absence of serum compared to presence of serum or IGF-1. Taken together, this data revealed that U0126 treatment decreased cell number in all cell lines and this effect was limited in the presence of IGF-1.

Since IGF-1R signaling is able to decrease U0126 sensitivity we next tested various strategies to down-regulate IGF-1R. As a first strategy we used siRNA mediated balanced IGF-1R downregulation that does not modify IGF-1R signaling [409, 410]. As a second strategy we downregulated IGF-1R with targeted antibodies (CP), inducing biased sustained β-arr mediated MAPK signaling [307]. The third strategy was to downregulate IGF-1R by Nutlin-3, inducing biased transient β-arr mediated MAPK signaling [411]. We identified that siRNA and CP downregulates IGF-1R in all cell lines while Nutlin-3 has effects only in DFB and to a lesser extent in BE cells. Only Nutlin-3 treated DFB cells indicated p53 re-activation. We tested the effect of treatments on IGF-1 induced proliferation of cells. We identified a decreased IGF-1 response in all siRNA, CP and Nutlin-3 treated DFB and to a lesser extend BE cells, whereas Mel28 were not affected. These results confirmed downregulation of IGF-1R and inhibition of IGF-1 response by three different strategies.

We have previously demonstrated that CP and Nutlin-3 are able to activate sustained or transient biased ERK activation [307, 411]. Therefore, we next tested the biased agonistic features of all three strategies. We treated cells with CP, Nutlin-3 and siRNAs, serum starved them and stimulated with IGF-1 for up to 60 minutes. WB analysis revealed the following patterns of pERK activation: in siRNA treated cells pERK1/2 was consistently decreased in all

cell lines compared to untreated controls; Nutlin-3 treatment induced an early increase and late decrease in the level of pERK1/2 in DFB and BE cells, compared to untreated controls; CP treatment decreased overall but sustained late pERK1/2 in all cell lines compared to untreated controls. These results indicated that all strategies decreased IGF-1R signaling, however siRNA treatment caused a balanced decrease, while Nutlin-3 and CP treatment induced biased transient and sustained pERK1/2, respectively.

We next investigated the effects of transient versus biased IGF-1R down-regulation on melanoma response to MEK1/2 inhibitor. Cells were first treated with siRNA, Nutlin-3 or CP, then with or without U0126. Results indicated a decrease in the level of IGF-1R in all cells except for Nutlin-3 treated Mel28 and an increase of p53 level only in Nutlin-3 treated DFB cells. The level of pERK was lowered in siRNA or Nutlin-3 combined with U0126 treated cells, but remained higher in CP combined with U0126 treated cells compared to control. Analysis for the total cell number indicated a 5-20% decrease after all three types of treatment. These data were combined with identical conditions of U0126 treatment alone to calculate a predicted additive response and compared with the observed response of combined treatment. We observed higher than predicted sensitivity to U0126 in all cells pre-treated with siRNA and in DFB and BE cells pre-treated with Nutlin-3, and close to predicted sensitivity to U0126 in cells pre-treated with CP. These data indicated synergy of balanced and transiently biased but not the sustained biased approach with MEK1/2 inhibition.

The main findings of this study are: 1) IGF-1R downregulation can be balanced or accompanied with transient or sustained biased signaling; 2) both balanced and transient biased IGF-1R downregulation synergizes with inhibition of MEK1/2, while sustained biased IGF-1R downregulation does not; 3) Nutlin-3 treatment offers a possible strategy to increase specificity of combined treatment.

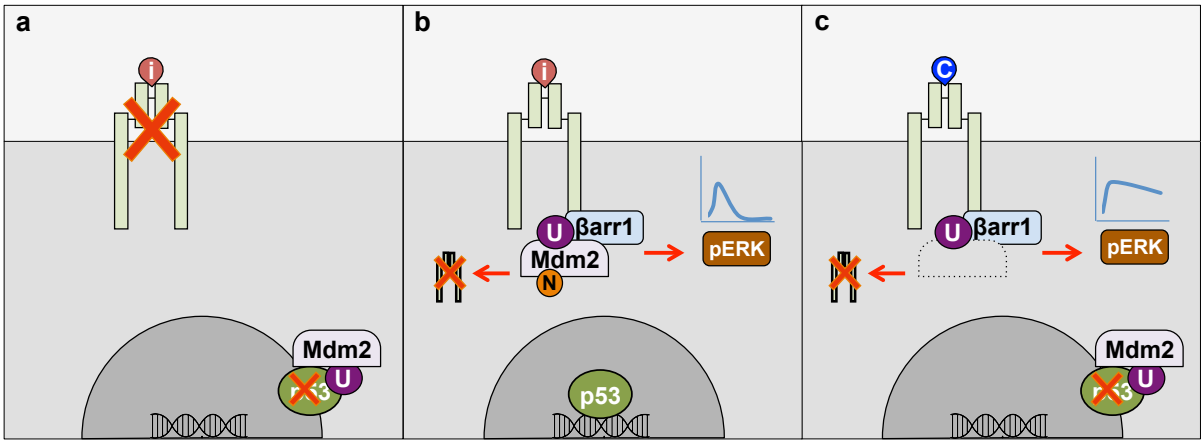

Figure 7. *Schematic illustration of IGF-1R and p53 activity after treatment with a) siRNAs against IGF-1R; b) p53 re-activator Nutlin-3; c) IGF-1R antibody CP*

3.4. Paper IV: Competing engagement of β-arrestin isoforms balance IGF-1R signaling and control response of melanoma cells to chemotherapy

Background and rationale:

Dacarbazine (DTIC) is a chemotherapeutic agent that induces DNA damage leading to activation of p53 pathway, followed by apoptosis and cell cycle arrest. Despite poor response and due to lack of alternatives it remains the main compound used for the treatment of metastatic melanoma. The IGF-1R is an important growth factor receptor involved in proliferation and invasion of melanoma cells [133, 397-399]. It been demonstrated recently that β-arrs control both mitogenic IGF-1R signaling and the tumor suppressor p53 pathway [272, 282, 392, 412]. In this study we aimed to investigate the possibility of co-targeting components of β-arr system in order to increase sensitivity to DTIC.

Conclusion and results:

We have demonstrated previously that β-arr isoforms play opposing roles in IGF-1 induced cell proliferation [412]. Thus, we first investigated the effect of β-arr modulation in melanoma cell lines. We first verified that all three melanoma cells express IGF-1R and the two β-arr isoforms. We next tested the effect of β-arr isoforms on melanoma cell proliferation and survival by down- and upregulating either β-arr with siRNA or β-arr encoding plasmids. We identified that β-arr1 silencing and β-arr2 up-regulation were most detrimental for cells and focused on these two conditions for further experiments.

Since β-arr isoforms also play opposing roles on IGF-1R signaling thus we next investigated the effect of β-arr1 silencing and β-arr2 overexpression on receptor signaling [412]. We transfected DFB and BE cells with siRNA against β-arr1 or β-arr2 encoding plasmid followed by serum starvation and IGF-1 stimulation for 0, 2, 5, 10, 30 and 60 minutes. Using WB analysis, we demonstrated that in both β-arr1 silenced and β-arr2 overexpressed cells level of phospho-ERK decreased at 30 and 60 min after stimulation. We further investigated the effect of β-arr modulation on proliferative/survival response of cells to IGF-1. Cells were serum starved after transfection, stimulated or not with IGF-1 for 24 h, followed by cell number analysis. Both β-arr1 downregulation and β-arr2 up-regulation inhibited IGF-1 response by decreased number of cells compared to unstimulated control. These results confirmed that a β-arr2 predominance in melanoma cells inhibits IGF-1 mediated pERK activation as well as an IGF-1 induced proliferative/survival response.

β-arr isoforms were demonstrated to induce different patterns of IGF-1R ubiquitination, followed by its degradation or endocytosis. It was also shown that IGF-1R internalization through β-arr1 or β-arr2 has opposing effects on the activation of p53 pathway [272, 282, 412]. Therefore, we next investigated the effect of β-arr modulation on IGF-1R degradation and activity of p53 pathway. Using WB analysis, we identified a decreased rate of IGF-1 induced receptor degradation in both β-arr1 silenced and β-arr2 upregulated cells. Importantly, in both conditions, the level of IGF-1R was decreased at time 0 compared to mock. WB analysis of lysates for p53 expression indicated its upregulation in both conditions. These data demonstrated that β-arr2 predominance downregulates ligand-free IGF-1R, inhibits ligand

induced IGF-1R degradation and activates p53 in melanoma cells.

DTIC is an alkylating agent that induces DNA damage causing cell cycle arrest and inhibition of melanoma cell growth [413]. Thus, we next tested the effect of DTIC as a single agent in BE, DFB and Mel28 melanoma cell lines. Cells were treated for 24 and 48 h with various doses of DTIC in media with or without serum or IGF-1. Using PrestoBlue we demonstrated decreased cell viability and in all cell lines. WB analysis of samples treated with different doses of DTIC for 24 h indicated an increase in the level of p53. DFB cells were more sensitive to DTIC and demonstrated a higher reactivation of p53 than in BE and Mel28 cells. These data confirmed the effect of DTIC on melanoma cells.

Since we demonstrated a negative effect of β-arr2 predominance on cell proliferation, we next tested if this can increase the sensitivity of melanoma cells to DTIC. We compared the effect of single and combined treatments of DTIC with β-arr1 silencing and β-arr2 overexpression. We calculated a predicted response to combined treatment by addition of two single agent sensitivity data. We observed a synergistic effect of combined treatments in all cell lines, indicating that β-arr2 predominance potentiates the DNA damage induced by DTIC in melanoma cells.

The main outcomes of this study are that 1) β-arr2 predominance positively affects p53 activation and negatively affects ligand induced IGF-1R degradation, MAPK activation and proliferation and survival in melanoma cells; 2) β-arr2 predominance synergizes with DTIC treatment in both mtp53 and wtp53 cells.

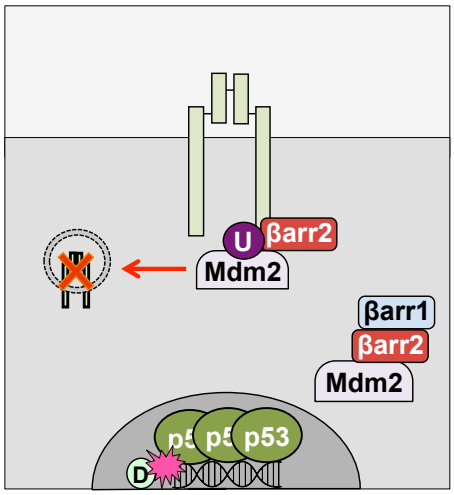

Figure 8. *Schematic illustration of the combined effects of dacarbazine with β-arr2 predominance*

4. DISCUSSION AND FUTURE PROSPECTIVES

The starting point of this thesis was that IGF-1R signaling and p53 pathway share similar regulatory mechanisms: Mdm2 and βarrs. This cross-regulation between two pathways involved in cancer has important implications for anti-cancer therapy and needs to be fully understood. The overall goal of the thesis was to investigate the IGF-1R/β-arr/Mdm2/p53 axis in cancer and to explore the potential use of its components as therapeutic targets.

In the first paper, we investigated the effect of destabilizing IGF-1R/Mdm2/p53 complex using Nutlin-3 and melanoma cells as an experimental model. In the second paper, we focused on the β-arr component of this axis and exploited the possibility to target these components in melanoma. In the third and fourth papers, we tried to exploit different approaches to target the IGF-1R/β-arr/Mdm2/p53 axis. In the third paper, we investigate the effect of co-targeting IGF-1R with MEK pathway, and in the fourth paper we explore the possibility of co-targeting β-arrs with a DNA repair pathway.

Our group has previously described various compounds and antibodies against IGF-1R as IGF-1R biased agonists [305-307]. Anti-IGF-1R antibody CP induces biased pERK that contributes to the survival of CP-treated Ewing's sarcoma cell lines [307]. The antimicrobial peptide LL-37 downregulates the IGF-1R whilst activating biased signaling, that contributes to breast cancer cells invasion and migration [306]. My studies reveal that Nutlin-3 also acts as an IGF-1R biased agonist, activating the MAPK pathway, that contributes to cell migration but not invasion. This study points out a highly specific mechanism of activation of IGF-1R biased signaling by various agonists, resulting in distinct biological outcomes. Characterization of the proteins involved in these pathways that could increase the possibility of more specific anti-cancer targeting is one of the future prospectives of this project. The involvement of β-arr isoforms in this signaling deserves particular attention. It has been demonstrated that β-arr1 mediates CP and LL-37 induced IGF-1R biased signaling [306, 307], however the role of β-arr isoforms in Nutlin-3 mediated ERK activation, as well as the specific role of β-arr2 in any types of IGF-1R signaling has not yet been identified.

In one of the studies we particularly focused on the β-arr components of the IGF-1R/β-arr/Mdm2/p53 axis. We revealed opposing roles of β-arr isoforms in the regulation of IGF-1R at multiple levels – β-arr1 induces while β-arr2 inhibits ligand-dependent IGF-1R degradation; β-arr1 sustains while β-arr2 shortens ERK activation; and β-arr2 activates, while β-arr1 inhibits p53 pathway downstream of IGF-1R activation. However, in the absence of IGF-1 both β-arrs downregulate IGF-1R with β-arr2 exerting a stronger effect. This points out that IGF-1R acts as both a Class A and Class B GPCR depending on the presence of the ligand. It seems that in the absence of the ligand IGF-1R preferentially and transiently recruits β-arr2, while an IGF-1 stimulated IGF-1R stably recruits β-arr1. Another important finding is that the antagonism between the two β-arr isoforms could be used as a target for anti-cancer therapy. We demonstrated that β-arr2 predominance inhibits response to IGF-1, causes cell cycle arrest, and activates p53 pathway resulting in a decrease of cell viability.

Opposing role of β-arrs at different levels of IGF-1R regulation raises the question of the reason for divergence of two isoforms with 78% identity and 88% similarity [414]. Possible

explanations include different affinity of the isoforms for distinct IGF-1R conformations, as well as competition for binding to the receptor and to each other [247, 392, 415, 416]. Another question is whether distinct agonists also induce different confirmations of IGF-1R, preferentially recruiting one of the isoforms. In this case biased agonists with preferential recruitment of β-arr2 would be favoured for anti-cancer treatment. Elucidation of the exact mechanism of IGF-1R interaction with β-arr isoforms is one of the future prospectives.

Investigation of the possibility to combine IGF-1R inhibitors with MEK inhibitors indicated that results are critically different depending on the strategy of IGF-1R inhibition. The first strategy of balanced IGF-1R inhibition by siRNA greatly enhanced response to MAPK inhibitors. However, a second strategy using anti-IGF-1R antibody CP was less successful. We revealed that CP not only induces biased IGF-1R signaling, but also is able to bias the IGF-1R towards its ligand even after CP removal. Thus, it was not surprising that CP treatment demonstrated limited effects on MEK1/2 inhibition. As discussed previously, certain RTK antibodies can bind to the receptor's extracellular domain without affecting receptor/ligand interaction [292-296]. Moreover, a specific IR antibody has been described that increases the binding affinity of insulin to the IR thus enhancing the receptors metabolic signaling [301]. This mechanism could be a possible explanation of the specific effect of CP on IGF-1R. The precise mechanism of CP/IGF-1R interaction remains to be investigated. A third strategy of IGF-1R inhibition using Nutlin-3 greatly enhanced the effect of MEK inhibitors. The transient character of Nutlin-3 induced biased signaling is an explanation for the better outcome. Thus, co-targeting MAPK pathway with IGF-1R using Nutlin-3 is the first anti-cancer strategy revealed in this project.

Another study was focused on the possibility to co-target β-arr components of the axis with the p53 pathway. We demonstrated that both β-arr1 inhibition and β-arr2 overexpression increase sensitivity of melanoma cells to DNA damage inducing drug dacarbazine. This study identified the second rational anti-cancer strategy in this project.

In conclusion, two successful strategies of co-targeting IGF-1R/β-arr/Mdm2/p53 axis with different cancer related pathways have been identified in this thesis. One strategy combines MEK inhibition with Nutlin-3 to greatly enhance IGF-1R degradation, transiently activate pERK and induce p53 re-activation. Another strategy is the combination inhibition of β-arr1 or activation of β-arr2 with dacarbazine treatment. This strategy, in contrast, has a moderate effect on IGF-1R degradation, strongly inhibits IGF-1R signaling and has double potential to activate p53 pathway. The synergistic effect of both strategies shows that accurate removal of multiple interconnected pathways of IGF-1R/β-arr/Mdm2/p53 axis can lead to an optimal context dependent anti-cancer effect.

5. ACKNOWLEDGEMENTS

To my main supervisor Leonard Girnita for making this thesis possible. Thanks for all of our discussions and for the freedom to have my own interpretations of things. Thanks for always having time to talk about science, but also being a friend and tolerating our half-joke complaints about the electric chair. In a little more than four years you have helped me to understand some fundamental principles of receptor pathology and cancer biology in general. This experience is priceless.

To my co-supervisor Klas Wiman, for giving me an opportunity to work on the p53 project and providing support whenever needed.

To Claire Worrall for introducing me to almost all basics of our research when I first joined the lab. To Iulian Oprea for all the early advice and teachings.

To Caitrin Crudden for making our work together fun, for language corrections, for invasion assays, statistic debates and for random fortune telling skills. Daniela Nedelcu for returning to our lab and teaching me genetic tools. Takashi Shibano, a wizard of immunoprecipitation, for all that I learned from you. To our talented exchange student Benjamin Gebhard for all the help with the drug assays. To external collaborators George Calin and Shin-Ichiro Takahashi. To our clinical team Ada Girnita, Stefan Seregard, Iara Drakensjo, Eric Trocme and to all other present and past members of the group.

To Padraig D'Arcy for the mentorship.

To members of the Wiman lab - Emarndeena, Susanne, Sofia, Vladimir for the materials and advice regarding the p53 part of this project. To other R8:4 corridor-mates - Satendra, Ahmed, Omid, Soniya, Christos etc for making us not feel alone any time in the lab.

To Catharina Svensson for all opportunities I had during my study in Uppsala University.

To my Uppsala-time friends - Anna, Katerina and Pierre for all their influence. To Laetitia for introducing me to GIMP and to the microwaves. Maxwell for books and for the typewriter. Rita for our adventures and dances. To my Vårberg-friends: Sunjay, Fadwa, Yogan, Michalis, Mike etc for all good times together. To my "community friends" - Karen, Natasha Sh, Natasha S, Vladimir, Galya, Nastya and others for our games, dinners and barbeques. To my old friends Tanya, Ayten, Katya, Fidan, Sonya and others for our long friendship.

To my Dad Tofik, to Arzu, to my brothers Ramin and Shamil, sisters-in-law Alma and Aysel and to my wonderful sibsprings Asiya, Timur and Zara. Thanks for your continuous support and happiness that you bring me. To my boyfriend Padraig for an enormous amount of IT-support and for everything else that has become easier and better since we met.

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